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PROVISIONAL PATENT APPLICATION

Field of the Invention:

5 This invention is related to use of cationic liposomes which are particularly useful for providing enhanced radiation sensitivity. The liposomes of the invention are particularly useful for delivery of antisense oligos.

Background of the invention:

10 It was previously reported that expression of antisense c-
15 raf-1 cDNA results in reduced expression (RNA) of c-raf-1 gene, a cause of delayed tumor growth in athymic mice and in enhanced radiation sensitivity of relatively radioresistant laryngeal squamous carcinoma cells, SQ-20B (Kasid et al., Science 243:1354-1356, 1989). These observations have been extended to examination of the effects of antisense raf oligodeoxyribo-
20 nucleotides (ATG-AS raf ODN) on the expression and activity of the product of c-raf-1 gene, Raf-1, in SQ-20B cells. Data suggests that ATG-AS raf ODN is a sequence-specific inhibitor of Raf-1 expression and activity, and tumor cells treated with this compound are significantly more sensitive to radiation as compared to the cells treated with sense-raf ODN or untreated tumor cells (Soldatenkov et al., Cancer J. Sci. Amer., 3:13-20, 1997).

Description of the Invention:

25 It is the purpose of this invention to provide a means of enhancing the effect of oligonucleotides by encapsulation in liposomes. The invention is exemplified using encapsulated raf

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oligodeoxyribonucleotides. The novel cationic liposomes of the invention were prepared using dimethyldioctadecyl ammonium bromide, phosphatidylcholine and cholesterol. These liposomes provide protection from degradation in plasma and normal tissues to protect the oligonucleotides while they are reaching their intended target cells. Hence, smaller amounts of oligonucleotides are needed to obtain desired results.

Cationic liposomes have been used to deliver genes in vitro and in vivo (Felgner, Editorial, Human Gene Therapy 7:1791-1793, 1996). The novel formulation of the cationic liposomes to encapsulate antisense raf oligonucleotides has been tested and found effective. It has been found that these liposomes encapsulate >90% oligos. Liposomal encapsulation provides protection of antisense raf oligonucleotide from degradation in plasma, and normal tissues, and that tumor cells treated with the liposome-encapsulated antisense raf oligo (LE-ATG-AS raf ODN) are significantly radiosensitive compared to control or sense raf oligo-treated cells. It is now disclosed herein that LE-ATG-AS raf ODN inhibits Raf-1 protein expression in solid tumors. (Gokhale et al., "Antisense 97: Targeting the Molecular Basis of Disease," Cambridge Symposium Meeting, May 1997). The liposomal compositions of the invention disclosed herein are believed to be particularly useful as radiosensitizers in solid tumors.

Materials and methods

Oligodeoxyribonucleotides

The sense and antisense raf ODNs were designed against the

translation initiation site of human c-raf-1 cDNA in accord with the teachings of Bonner (Bonner et al., Nucleic Acids Res., 14:1009-1015, 1986), and have the following sequence: sense ODN (ATG-S raf), 5'-GCATCAATGGAGCAC-3'; antisense ODN (ATG-AS raf), 5'-GTGCTCCATTGATGC-3'. Only two of the bases, one at each end, are phosphorothioated. The ODNs were synthesized as described earlier (Soldatenkov et al., Cancer J Sci Amer, 3:13-20, 1997).

Liposome preparation

Liposome-encapsulated raf oligodeoxyribonucleotides, LE-ATG-S raf ODN and LE-ATG-AS raf ODN, were prepared using dimethyldioctadecyl ammonium bromide, phosphatidylcholine and cholesterol (Avanti Polar Lipids, Inc., Alabaster, AL, USA) in a molar ratio of 1:3.2:1.6. Briefly, the lipids dissolved in chloroform or methanol were evaporated to dryness in a round-bottomed flask using a rotatory vacuum evaporator. The dried lipid film was hydrated overnight at 4°C by adding 1 ml of ODN at 1.0 mg/ml in phosphate buffered saline (PBS). The film was dispersed by vigorous vortexing and the liposome suspension was sonicated for 5 min in a bath type sonicator (Laboratory Supplies Co. Inc., Hicksville, NY, USA). The ODN to lipid ratio was 30 µg ODN/mg of lipid. The unencapsulated ODN was removed by washing the liposomes by centrifugation (3 times at 75,000 g for 30 min) in PBS. The encapsulation efficiency was determined by the scintillation counting of an aliquot of the preparation in which traces of ³²P-end labeled ODN were added to the initial ODN. The entrapment efficiency was found to be >90% (n=10). The liposome

encapsulated ODN were stored at 4°C and used within 2 weeks of preparation. Blank liposomes were prepared exactly as described above but without ODN.

5 Animals

Male Balb/c nu/nu mice, 10-12 weeks old, were maintained in the RRF facility of the Georgetown University according to accredited procedure and fed purina chow and water ad libitum.

Pharmacological disposition studies

10 The pharmacological disposition of free (ATG-AS) or liposome-encapsulated antisense raf oligodeoxyribonucleotide (LE-ATG-AS) was carried out in Balb/c nu/nu mice. Male Balb/c nu/nu mice were injected intravenously via tail vein with 30 mg/kg of ATG-AS raf ODN or LE-ATG-AS raf ODN. At 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and 48 h after injection, one animal in each group was bled from the retro-orbital sinus into heparinized tubes and sacrificed by cervical dislocation. The blood was centrifuged immediately at 2000 r.p.m for 10 min at 4°C to separate the plasma. The liver, spleen, kidney, heart and lung were rapidly excised and rinsed in ice-cold normal saline. The organs and plasma were stored frozen at -70°C until analysis.

20 ODN was isolated from plasma samples using the phenol/chloroform extraction method, and from tissues using a DNA extraction kit (Stratagene, La Jolla, CA, USA). The extracts were then loaded onto 20% polyacrylamide/8 M urea gels and electrophoresed in TBE buffer. The gel was electroblotted onto nylon membrane in

0.5X TBE buffer at 20 V for 1 h. The blots were probed with ³²P-labeled sense raf ODN (ATG-S raf ODN) in Quickhyb buffer (Stratagene, La Jolla, CA, USA) at 30°C overnight. The ODN concentration standard was prepared by spiking known amount of the ATG-AS raf ODN in blank plasma or blank tissue samples, followed by extraction as described above. The autoradiographs were scanned using a computer program (ImageQuant software version 3.3, Molecular Dynamics), and the amounts of ATG-AS raf ODN in various samples were calculated by comparison to standards.

Cell culture

SQ-20B tumor cells were established from a laryngeal squamous cell carcinoma of a patient that had failed a full course of radiation therapy (Weichselbaum, et al., *Proc. Natl. Acad. Sci.*, 83: 2684-2688 (1986)). Tumor cells were grown as monolayers in Dulbecco's modified Eagle's medium (GIBCO/BRL, NY, USA) supplemented with 20% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM non-essential amino acids, 0.4 µg/ml hydrocortisone, 100 µg/ml streptomycin and 100 U/ml penicillin.

Raf-1 immunoprecipitation and immunoblotting assays

Logarithmically growing SQ-20B cells were exposed to LE-ATG-AS raf ODN, LE-ATG-S raf ODN or blank liposomes for various time intervals and varying concentrations in 1% serum containing medium. Following incubation, cells were lysed in buffer containing 500 mM HEPES (pH 7.2), 1% NP-40, 10% glycerol, 5 mM

sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μ g/ml aprotinin, and 20 μ g/ml leupeptin. The lysates were clarified by centrifugation at 16,000 g for 20 min and the protein concentration was determined (Pierce, Rockford, IL, USA).

5 Raf-1 was immunoprecipitated from whole cell lysates, normalized for protein content, using protein A-agarose conjugated with rabbit polyclonal antibody against 12 carboxy terminal amino acids of human Raf-1 p74 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The immunoprecipitates were washed once with
10 lysis buffer, then with 0.5 M LiCl-100 mM Tris-HCl, pH 7.4 and finally with 10 mM Tris-HCl, pH 7.4. For immunoblotting, the immune complexes were boiled in Lammeli sample buffer and resolved by 7.5% SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membrane and detection with polyclonal anti-Raf-1 antibody and ECL reagents according to manufacturer's protocol (Amersham Corp., Arlington Heights, IL, USA). Raf-1 protein expression was quantified by a computer program (Image-Quant, Molecular Dynamics).

Raf-1 protein kinase activity assay

20 Logarithmically growing SQ-20B cells were treated with 10 μ M LE-ATG-AS raf ODN, LE-ATG-S raf ODN or blank liposomes for 8 hours in 1% serum containing medium. Cells were lysed as described above, and lysates, normalized for protein content, were immunoprecipitated with agarose-conjugated anti-Raf-1
25 antibody overnight at 4°C. Raf-1 phosphotransferase activity was assayed in vitro using mitogen-activated protein kinase kinase

(MKK1) as substrate (kindly provided by Dr. Sturgill's laboratory) in kinase buffer containing 30 mM HEPES (pH 7.4), 1 mM manganese chloride, 1 mM DTT, 0.1 mM ATP, and 20 μ Ci [λ - 32 P]ATP (6,000 Ci/mmol) as described before (Kasid *et al.*, Nature 382, 813-816, 1996). Radiolabeled reaction products were separated by 10% SDS-PAGE and autoradiographed. The MKK1 bands were quantified as described above.

Radiation survival dose response assay

The appropriate number of SQ-20B cells were seeded in T25 tissue culture flasks (Corning, NY, USA) in medium containing 20% FBS, and allowed to attach for 8 hours at 37°C. The medium was replaced with medium containing 1% FBS and the cells were then exposed to 10 μ M LE-ATG-AS raf ODN, LE-ATG-S raf ODN or blank liposomes for 6 h. The cells were then irradiated with 137 Cs (JL Shepard MARK I irradiator) using various doses at a dose rate of 114 cGy/min, followed by incubation for 2 h. The medium in all flasks was then replaced with 20% FBS containing medium and the cells were incubated for 7-10 days. Surviving colonies were fixed and stained with 0.5% methylene blue - 0.13% carbol fuchsin in methanol solution. Colonies containing 50 or more cells were scored and data were fitted to the Albright's computer-generated single-hit multitarget and linear-quadratic models of radiation survival response.

SQ-20B tumor xenograft studies

Logarithmically growing SQ-20B cells (2×10^6) were injected subcutaneously in the flank region on both sides in male Balb/c

nu/nu mice under mild anesthesia. Tumors were allowed to grow to a mean tumor volume of 115 mm³ before initiation of ODN treatment.

For intratumoral delivery of LE-ATG-AS raf ODN or LE-ATG-S raf ODN, mice were randomly divided into 3 groups. Three mice in each group received intratumoral injections of 4 mg/kg LE-ATG-AS raf ODN on the right flank, and LE-ATG-S raf ODN on the left flank. The ODN was administered intratumorally daily for 7 days. Control groups received normal saline or blank liposomes. Mice were sacrificed 24 hours after the last dose of ODN, and the organs were rapidly excised, rinsed in ice-cold normal saline and stored at -70°C until analysis. Raf-1 protein expression was analyzed from tissue homogenates by immunoprecipitation and immunoblotting and quantified using the ImageQuant computer program as described above.

Results

Liposomal encapsulation protects ATG-AS raf ODN *in vivo*

The plasma pharmacokinetics of LE-ATG-AS raf ODN in presented in Figure 1. The plasma concentration-time profile of LE-ATG-AS raf ODN. 30 mg/kg LE-ATG-AS raf ODN (top panel) or ATG-AS raf ODN (middle panel) was administered i.v. in Balb/c nu/nu mice. Blood samples were collected from retro-orbital sinus at indicated times after injection and the ODN in plasma samples was extracted by phenol:chloroform. The samples were electrophoresed on 20% polyacrylamide/8 M urea gel and electroblotted on nylon membrane. The blots were probed with ³²P-labeled

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ATG-S raf ODN. Autoradiographs were scanned using a computer program (ImageQuant software version 3.3, Molecular Dynamics). St, standard prepared by spiking known concentration of ATG-AS raf ODN in blank plasma. Top panel: Samples were diluted prior to electrophoresis as follows: lane 1, 12x; lanes 2 and 3, 4x; lanes 4 and 5, 3.3x; lane 6, 2x; lane 7, 1.4x; lane 8, 1.3x; and lane 9, 1x. St lanes represent 0.25, 0.5 and 1.0 $\mu\text{g/ml}$ of ATG-AS raf ODN. Middle panel: Samples were diluted prior to loading as follows: lane 1, 4x; lanes 2 and 3, 2x; lanes 4, 5 and 6, 1x; lane 7, 0.75x; lanes 8 and 9, 0.6x. St lanes represent 0.125, 0.25, and 0.5 $\mu\text{g/ml}$ of ATG-AS raf ODN. Bottom panel: Plasma concentration-time curve of LE-ATG-AS raf ODN shown in the top panel. Quantification data were calculated based on a known concentration of the standard sample, and then normalized against dilution factors at various time points.

Following intravenous administration, the peak plasma concentration of 6.39 $\mu\text{g/ml}$ was achieved and intact ODN could be detected upto 24 h. The decrease in plasma concentration of LE-ATG-AS raf ODN followed a biexponential pattern with an initial half-life ($t_{1/2\alpha}$) of 24.5 min and a terminal half-life ($t_{1/2\beta}$) of 11.36 h. The area under the plasma concentration-time curve for LE-ATG-AS raf ODN was 5.99 $\mu\text{g.h/ml}$, with total body clearance of 75.94 ml/min/kg and volume of distribution of 74.67 L/kg . In contrast, intact free ODN (ATG-AS raf ODN) was detectable only at 5 min; the plasma concentration being 9.75 $\mu\text{g/ml}$. These observations indicate that ATG-AS ODN was either rapidly cleared

from the circulation, or extensively degraded in plasma due to nuclease activity.

The normal tissue distribution of LE-ATG-AS raf ODN is presented in Figure 2. Normal tissue distribution profiles of LE-ATG-AS raf ODN. Tissue samples were collected at indicated times after i.v. administration of 30 mg/kg LE-ATG-AS raf ODN. ODN was extracted from homogenized tissues using a DNA extraction kit (Stratagene). Samples were electrophoresed and electroblotted. The blots were probed with ³²P-labeled ATG-S raf ODN and autoradiographs were analysed as explained in the legend to Figure 1. Panel A: Representative autoradiographs from liver (top) and kidney (bottom). Panel B: ATG-AS raf ODN levels in different tissues at indicated times after a dose of 30 mg/kg LE-ATG-AS raf ODN was administered i.v. Quantification data were calculated based on a known concentration of the standard sample, and then normalized against the weight of the tissue sample collected.

Intact ODN could be detected in all organs examined upto 48 h. Interestingly, following ATG-AS raf ODN administration, intact ODN could be detected only at 5 min in all the organs examined and degradative products were observed at all other times (data not shown). These findings, along with the plasma data for LE-ATG-AS raf ODN, suggests that ODN with only the end bases phosphorothioated is rapidly degraded in vivo and that liposome encapsulation protects it from degradation for at least 48 h.

Specificity of inhibition of Raf-1 protein expression and activity in vitro

Initially, the possibility of cytotoxic effects of liposomes in SQ-20B cells was examined. Blank liposomes, at concentration equivalent to 10 μ M LE-ATG-AS raf ODN, were found to be non-cytotoxic as determined by the clonogenic and trypan blue dye exclusion methods (data not shown). However, blank liposomes showed cytotoxicity at doses higher than 20 μ M, therefore, a dose of 10 μ M or less was used for in vitro experiments. Further, 10 μ M LE-ATG-AS raf ODN or LE-ATG-S raf ODN was non-toxic to SQ-20B cells (data not shown).

The effects of LE-ATG-AS raf ODN on Raf-1 protein expression and activity are shown in Figures 3 and 4. Referring to Figure 3, specificity of inhibition of Raf-1 protein expression by LE-ATG-AS raf ODN. Panel A: Time-course analysis. Logarithmically growing SQ-20B cells were treated with 10 μ M LE-ATG-AS raf ODN (AS), 10 μ M LE-ATG-S raf ODN (S) or blank liposomes (BL) for indicated time in 1% FBS containing medium. Untreated control cells (C) were simultaneously switched to 1% FBS containing medium for 8 h. Whole cell lysates were normalized for total protein content and immunoprecipitated with agarose-conjugated polyclonal anti-Raf-1 antibody (Santa Cruz). Immune-complexes were resolved on 7.5% SDS-PAGE and Raf-1 protein expression was detected by immunoblotting with polyclonal anti-Raf-1 antibody (Santa Cruz), followed by the ECL detection protocol (Amersham)

(top). Results from three independent experiments were quantified using a computer program (ImageQuant, Molecular Dynamics), and data are expressed relative to the level of Raf-1 in LE-ATG-S raf ODN-treated cells (bottom). Panel B: Dose-response analysis. Logarithmically growing SQ-20B tumor cells were treated with indicated concentrations of LE-ATG-AS raf ODN (AS) or LE-ATG-S raf ODN (S) in 1% FBS containing medium for 8 h. Normalized cell lysates were analyzed for Raf-1 protein expression (top). Quantification data from three independent experiments are expressed as the level of Raf-1 protein expression in LE-ATG-AS raf ODN-treated cells relative to LE-ATG-S raf ODN-treated cells (bottom).

Referring to Figure 4, Inhibition of Raf-1 protein kinase activity by LE-ATG-AS raf ODN. Logarithmically growing SQ-20B cells were treated with 10 μ M LE-ATG-AS raf ODN (AS), or 10 μ M LE-ATG-S raf ODN (S) for 8 h in 1% FBS containing medium. Control cells (C) were simultaneously switched to 1% FBS containing medium for 8 h. Whole cell lysates were normalized for protein content, and Raf-1 was immunoprecipitated as described in the legend to Figure 3. Phosphotransferase activity of Raf-1 in immune-complexes was assayed in vitro using a physiologic substrate MKK1. Radiolabeled reaction products were separated by electrophoresis and autoradiographed (inset). Quantification data from two independent experiments each performed in duplicate are expressed as Raf-1 enzymatic activity in LE-ATG-AS raf ODN-treated cells relative to LE-ATG-S raf ODN-

treated cells.

Time-course experiments revealed that a maximum inhibition (52.3 ± 5.7%) of Raf-1 protein expression (~ 74kDa) occurred at 8 h post-incubation of cells with 10 μM LE-ATG-AS raf ODN (Figure 3A). The inhibitory effect of LE-ATG-AS raf ODN was maintained upto 24 h (45.6 ± 9.8%). The level of Raf-1 protein was comparable in the control untreated cells (C), blank liposome-treated cells (BL), and LE-ATG-S ODN-treated cells (Figure 3A), indicating the LE-ATG-AS raf ODN specifically inhibited the Raf-1 protein expression in SQ-20B cells. Dose response studies showed that 35.94 ± 16.8% and 52.3 ± 5.7% inhibition of Raf-1 expression occurred with 5 μM and 10 μM LE-ATG-AS raf ODN treatment for 8 h, respectively (Figure 3B).

The effects of LE-ATG-AS raf ODN on the enzymatic activity of Raf-1 protein kinase using mitogen-activated protein kinase kinase (MKK1) as substrate were studied. In concurrence with Raf-1 protein inhibition data, it was found that 10 μM LE-ATG-AS raf ODN treatment for 8 h inhibited 62.6 ± 9.0% in vitro phosphotransferase activity of Raf-1 protein (Figure 4). LE-ATG-S raf ODN did not have any effect on the Raf-1 protein kinase activity as compared with the untreated control cells.

LE-ATG-AS raf ODN is a biological radiosensitizer

Radiation survival dose responses of SQ-20B cells exposed to LE-ATG-AS raf ODN, LE-ATG-S raf ODN, and blank liposomes are presented in Figure 5 and Table 1. Comparison of the radiation survival dose response of SQ-20B cells treated with LE-ATG-AS raf

ODN (AS), LE-ATG-S raf ODN (S), or blank liposomes (BL). The appropriate number of cells were seeded in duplicate to obtain 40-60 colonies per T-25 flask (Costar) for each radiation dose. The clonogenic survival data were computer-fitted to the single-hit multitarget model of radiation survival dose response. Representative data from one experiment performed for each treatment category are shown.

The plating efficiencies of cells treated with S/AS ODN or blank liposomes were comparable (Table 1). Radiation survival dose responses of the blank liposome-treated (BL) and LE-ATG-S raf ODN-treated cells were also comparable (Figure 5). LE-ATG-AS raf ODN treatment resulted in a significant radiosensitization (Table 1). Based on a ratio of the mean inactivation dose, the dose modifying factor (DMF) of LE-ATG-AS raf ODN treatment was ~1.6. Significant decreases observed in the values of radiobiological parameters, \bar{D} , D_q , and D_0 of SQ-20B cells following treatment with the LE-ATG-AS raf ODN indicate a good correlation between the DNA sequence-specific inhibition of Raf-1 protein kinase and the radiosensitization of these relatively radioresistant tumor cells.

LE-ATG-AS raf ODN is a specific inhibitor of Raf-1 protein expression in solid tumor

The effects of intratumoral administration of LE-ATG-AS raf ODN and LE-ATG-S raf ODN on the expression of Raf-1 protein was examined in a SQ-20B tumor xenograft model. Mice with tumors on both flanks received intratumorally LE-ATG-AS raf ODN on the

right flank, and LE-ATG-S ODN on the left flank. Results shown in Figure 6 demonstrate a significant inhibition of Raf-1 protein in tumor tissue following treatment with LE-ATG-AS raf ODN compared with LE-ATG-S raf ODN ($60.3 \pm 6.4\%$). Inhibition of Raf-1 protein expression by LE-ATG-AS raf ODN in SQ-20B tumor xenografts. SQ-20B tumors were established subcutaneously in both hind limbs of nude mice, Balb C nu/nu (mean tumor volume 115 mm^3). Each animal then received intratumoral injections of LE-ATG-AS raf ODN (AS) on the right flank and LE-ATG-S raf ODN (S) on the left flank at a dose of 4 mg/kg daily for 7 days. Tumor tissue was excised 24 h after the last treatment, and Raf-1 protein expression in tumor samples was analyzed as explained in the legend to Figure 3. Representative data from two animals are shown (inset). ECL images were quantified using a computer program (ImageQuant, Molecular Dynamics), and quantification data from three mice are expressed as the level of Raf-1 protein expression in LE-ATG-AS raf ODN-treated tumors relative to LE-ATG-S raf ODN-treated tumors.

Liposomes prepared in accord with the teachings of the invention are non-toxic both in culture and in animals. While only specific liposomes are disclosed herein, the novel carriers may be used for delivery of a variety of DNA-based compounds for delivery.

Table 1. Radiation Survival Parameters of SQ-20B Cells Treated with LE-ATG-S/AS Raf ODN

Raf ODN	No. of Expts.	D ₀ (Gy)	D _q (Gy)	\bar{n}	α (Gy ⁻¹)	β (Gy ⁻²)	\bar{D} (Gy)
Blank liposomes/	5	2.795	1.445	2.012	0.2184	0.0087	3.659
LE-ATG-S*		± 0.38	± 1.22	± 1.34	± 0.11	± 0.00	± 0.02
LE-ATG-AS	3	2.287	0.051	1.021	0.4385	0.0000	2.280
		± 0.23	± 0.05	± 0.19	± 0.05	± 0.00	± 0.00

The appropriate number of cells were seeded in duplicate T25 flasks per dose in each experiment. Plating efficiencies of the blank liposome-treated, LE-ATG-S Raf ODN-treated, and LE-ATG-AS Raf ODN-treated cells were in the range of 65-79%, 52-83%, and 59-90% respectively. Clonogenic survival data were computer-fitted to the single-hit multitarget and the linear-quadratic models of radiation survival dose response.

*Composite values of the various parameters were obtained from the three experiments performed with LE-ATG-S Raf ODN-treated cells and two experiments performed with the blank liposome-treated cells.

The liposomes of the invention provide significant protection of antisense oligonucleotides against degradation in blood and normal tissue. The formulation may replace the need for complete modification of all bases of the antisense oligonucleotides for therapeutic uses. Compositions comprising oligonucleotides may be administered in many ways, depending on the target tissue.

The particular method used to deliver compositions of the invention to the tissues of the intact animal will depend on the particular tissue to which it is administered. For example, compositions of the invention can be administered intrathecally to facilitate contact of the active agent with neuronal tissue. Liposomal compositions may also be administered to tissue locally during surgery. The liposomes may also be injected into the target tissue. When the target tissue is the lining of a hollow organ, they may be introduced into the lumen of the organ.

The dosage required will depend on the agent and the subject being treated with the liposomal compositions. For example, when a radiosensitizing oligonucleotide is administered by means of liposomes, a radiosensitizing amount of oligonucleotides must reach the target organ.

What we claim is:

1. A composition comprising a cationic liposome of dimethyldi-
octadecyl ammonium bromide, phosphatidylcholine and cholesterol.

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2. A composition of claim 1 wherein the liposome contains an
antisense oligonucleotide sequence.

3. A composition of claim 2 wherein the antisense sequence is
a raf oligodeoxynucleotide.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Masid, et al
USPN Not assigned
Filed: March 21, 1997
For: LIPOSOMES CONTAINING OLIGONUCLEOTIDES

DECLARATION CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9 (f) & 1.27(d))--NONPROFIT ORGANIZATION

Hon. Commissioner of Patents and Trademarks
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LIPOSOMES CONTAINING OLIGONUCLEOTIDES

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and belief are believed to be true; and further that those state-
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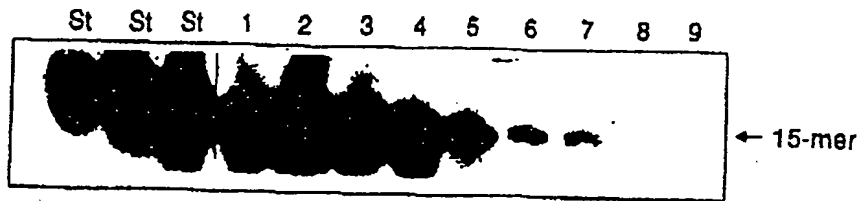
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LE-ATG-AS:



ATG-AS:

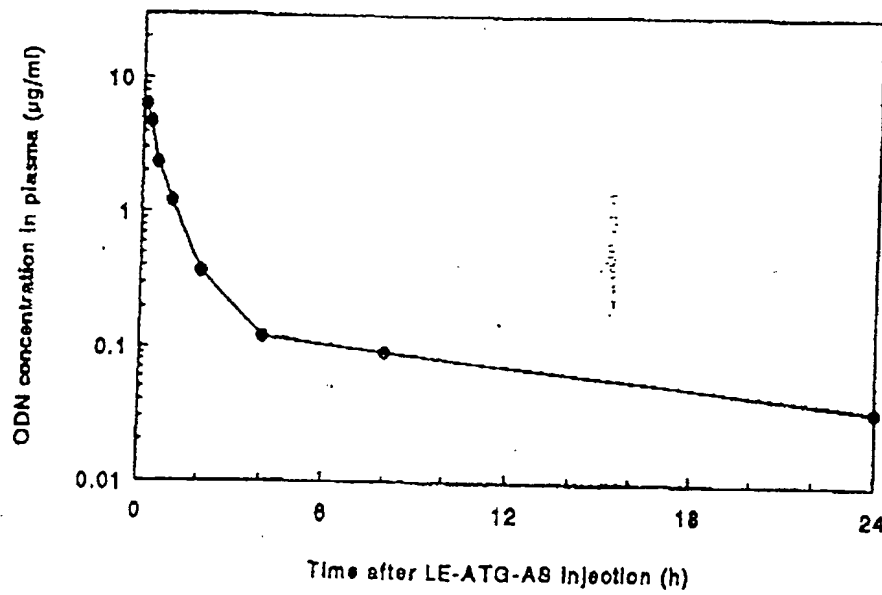
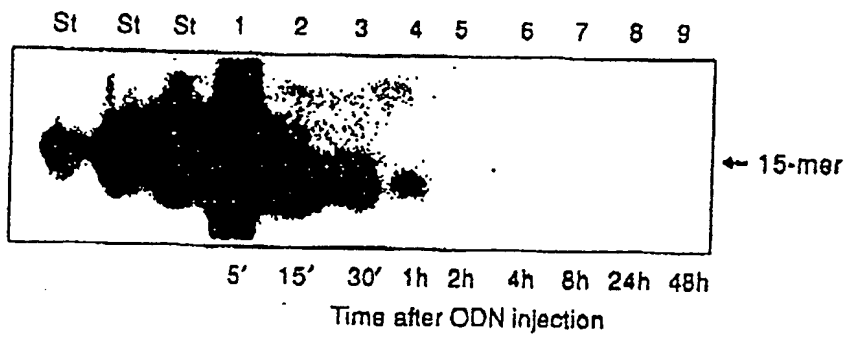
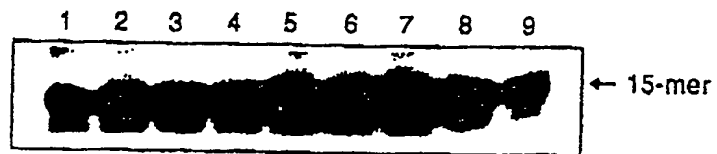


Fig 1

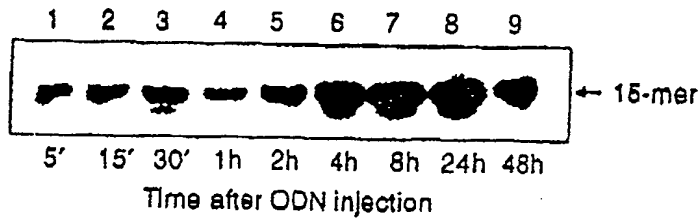
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A

LIVER:



KIDNEY:



B

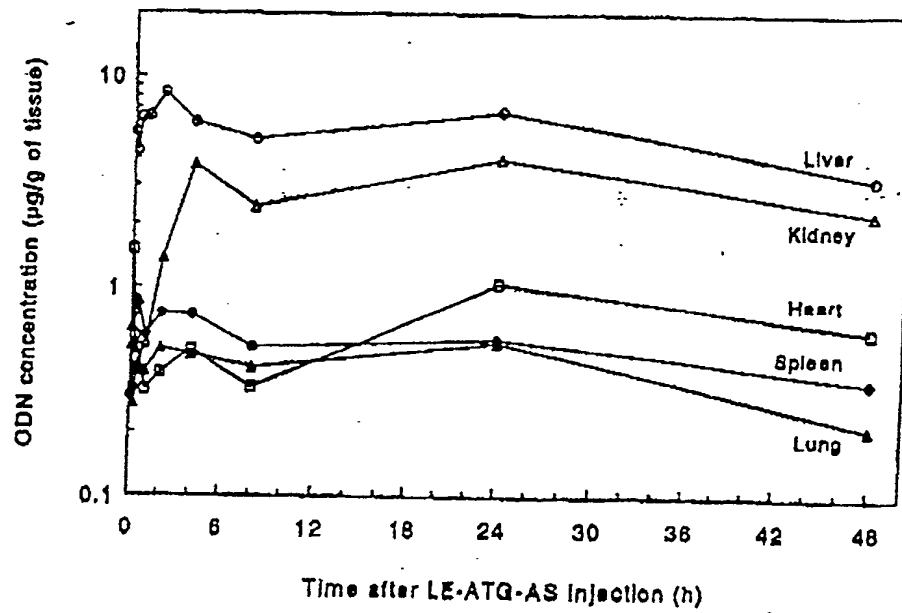


Fig 2

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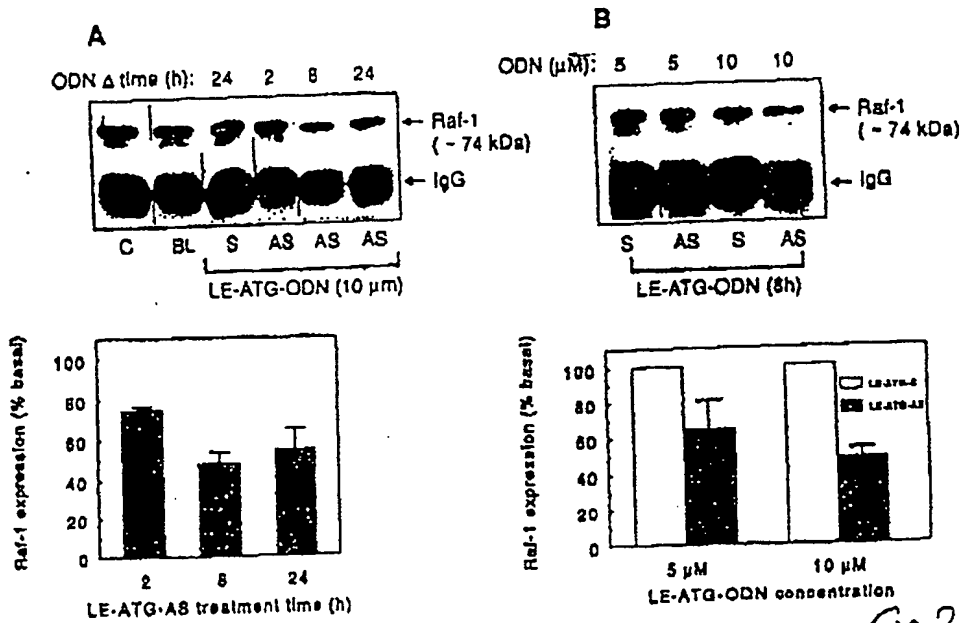


Fig 3

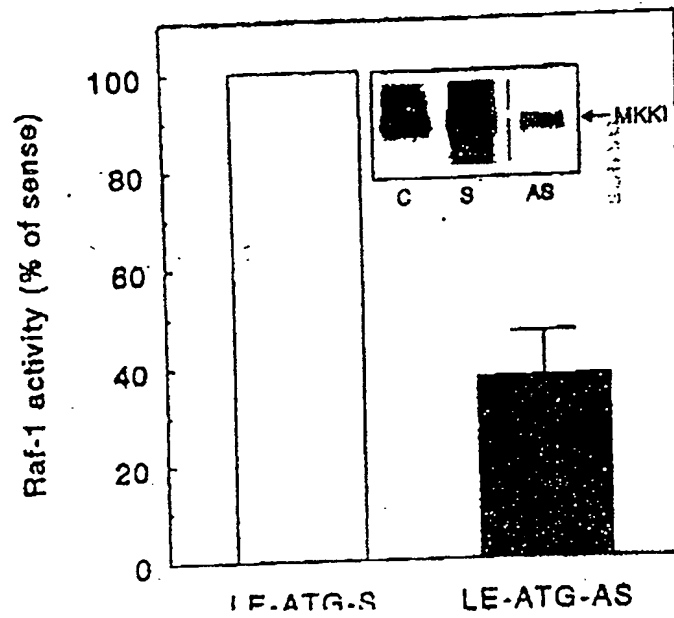


Fig 4

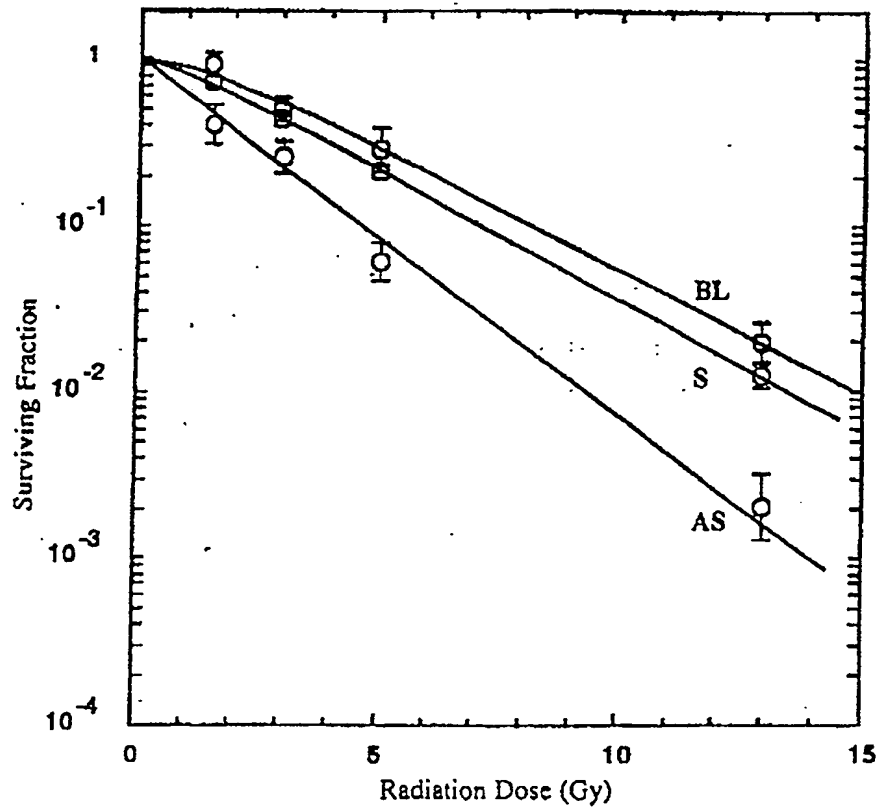


Fig 5

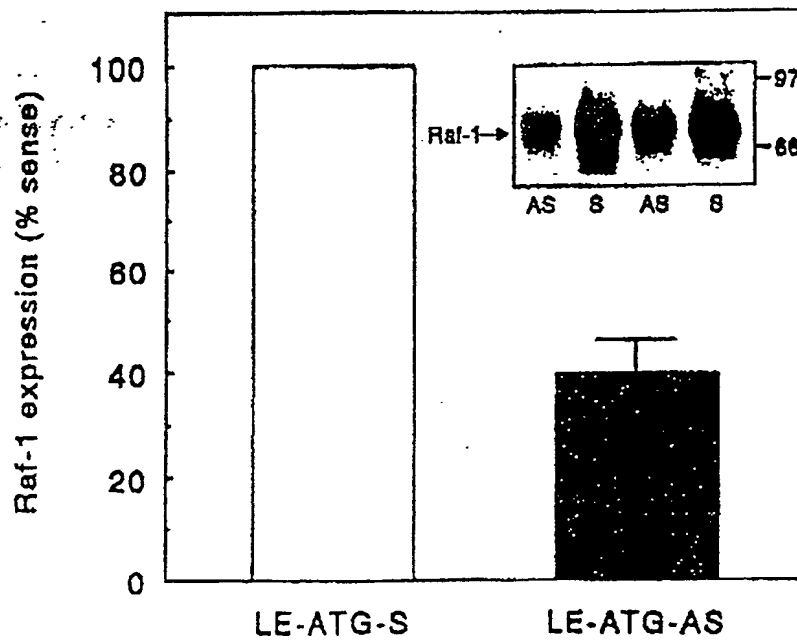
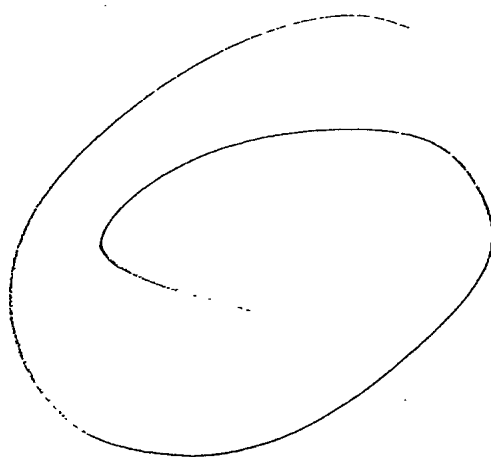


Fig 6



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